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Iron stress in the Pacific region of the Southern Ocean: evidence from enrichment bioassays

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ABSTRACT: The effect of iron on phytoplankton physiology in a scarcely investigated part of the Pacific region of the Southern Ocean was studied during an expedition aboard RV 'Polarstern'. Physiological effects of iron enrichments were studied during short-term bottle experiments (72 h) in high-nutrient, low-chlorophyll (HNLC) water samples. Throughout the expedition (March to May 1995), surface water nitrate concentrations were high (21 to 27 µM) and surface water silicate concentrations varied considerably north (<3.5 µM) and south (>13 µM) of the Polar Front, whereas low chlorophyll *a* concentrations (<0.2 µg l^{-1}) were measured in the research area. Small-sized cells (<5 µm) dominated the phytoplankton community; diatoms were observed only occasionally north of the Polar Front. Total phytoplankton numbers, as determined by flow cytometry, ranged from 1500 to 8000 cells ml^{-1}. Ambient dissolved iron concentrations in the experimental area were low and ranged from 0.1 to 0.4 nM in surface waters. In the experimental series, indications of iron stress in the indigenous phytoplankton assemblage were found. Whereas the species composition remained virtually unaltered upon addition of 2 nM iron, significant increases in nitrate uptake and enhanced nitrate reductase activities were observed within 32 h after the start of the experiments. Ammonium uptake, which prevailed over nitrate uptake, remained unaltered by the iron additions. In addition to the physiological effects, a molecular marker of iron stress, flavodoxin, was detected in phytoplankton protein extracts collected after 72 h of incubation. Irrespective of iron deficiency, silicate concentrations in the northern part of the research area were likely below half-saturation values for growth for diatoms and thereby likely prevented diatom blooms. The results of this study indicate that the nano-phytoplankton in the Pacific region of the Southern Ocean are iron stressed; upon iron enrichment, a physiological stimulation is observed. Our results support a lack of iron as one of the causes of HNLC conditions in this region.

KEY WORDS: Iron stress · Nitrogen assimilation · Phytoplankton · Flavodoxin

INTRODUCTION

In recent years, oceanographers have focused much attention on the role of iron in limiting phytoplankton productivity and biomass potential in large regions of the world's oceans (Chisholm & Morel 1991, Bruland & Wells 1995). These areas, known as high-nutrient, low-chlorophyll (HNLC) regions, are characterized as having low surface water levels of iron (<0.5 nM dissolved Fe), but otherwise are apparently replete with major nutrients such as nitrate and phosphate (Sunda 1989, Bruland et al. 1991, de Baar 1994, Cullen 1995, de Baar et al. 1995, 1997). Research has focused on the 3 regions well known for their HNLC conditions, notably the equatorial Pacific Ocean (Martin & Fitzwater 1988, Price et al. 1991, 1994), the subarctic Pacific Ocean (Boyd et al. 1996, LaRoche et al. 1996) and the Atlantic region of the Southern Ocean (de Baar et al. 1990,

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1995, 1997, Buma et al. 1991, Scharek et al. 1997, van Leeuwe et al. 1997). The Pacific region of the Southern Ocean has received comparatively little or no attention for possible iron limitation.

The 'ecumenical' Fe hypothesis (Morel et al. 1991a) is most frequently invoked to explain the observed phenomenon of low chl *a* concentrations in otherwise high-nutrient areas. It is thought that the small phytoplankton (<10 µm) are less affected by low iron concentrations, using ammonium as their N source (regenerated production) and limited in their numbers through efficient micro-zooplankton grazing. The rare, larger phytoplankton species (>10 µm), using nitrate as their N source (new production), are deemed to be more severely Fe limited. Upon addition of iron to the system, physiological constraints imposed on the latter species are relieved, biomass increases and removal of major nutrients commences. In this description it is assumed that the small phytoplankton are not Fe limited and grow at values near the maximum growth rate (HNLC conditions = 'grazer controlled phytoplankton populations in an iron-limited ecosystem', cf. Price et al. 1994). This is disputed by Greene et al. (1994) and, most recently, by Behrenfeld et al. (1996), who studied variability in the quantum yield of fluorescence of phytoplankton photosynthesis and postulate that the indigenous phytoplankton in the equatorial Pacific Ocean are Fe limited. Similarly, Zettler et al. (1996) observed physiological effects on most phytoplankton cells under low iron conditions in the equatorial Pacific. This controversy may be the result of different approaches used to determine the iron nutritional status; sensitive, relevant physiological parameters must be sampled on relevant time scales. It is generally accepted that, in bottle experiments, a physiological response to iron addition must be measured within 1 to 2 d; after 3 to 5 d, only ecological responses can be studied (Geider & LaRoche 1994).

One may speculate that only trace amounts of aeolian iron are introduced into the system in the Pacific region of the Southern Ocean. The dominant currents circle the Antarctic continent in a clockwise manner, there is a lack of meridional boundaries, and westerly winds, hardly ever touching land masses, prevail (Duce & Tindale 1991, Gargett 1991).

The subject of the present study is the effect of iron enrichment on the phytoplankton community in the Pacific region of the Southern Ocean. Short-term iron enrichment bioassays were performed. In order to minimize bottle artefacts (Geider & LaRoche 1994), the incubations lasted no longer than 72 h. During this period, the effects of iron enrichments (2 nM) on phytoplankton N metabolism (nitrate and ammonium uptake and nitrate reductase activity) were tested in order to address the question of whether phytoplankton physiology is limited by iron. Furthermore, a biomarker of iron stress, flavodoxin (La Roche et al. 1995, 1996, McKay et al. 1997), was tested. Nutrient consumption, cell numbers, size classes and chl *a* were monitored. The effects of iron enrichment on chromatic adaptation are described elsewhere (van Leeuwe et al. 1998b in this issue). In combination with shipboard Fe measurements (de Baar et al. in press), taxonomic classification using photopigments (van Leeuwe et al. 1998a) and iron speciation measurements (Nolting et al. in press), a comprehensive approach towards a better understanding of iron-phytoplankton interactions was carried out.

METHODS

Iron enrichment bioassays were performed during the ANT XII/4 expedition (March 21 to May 14, 1995, austral summer) aboard RV 'Polarstern' from Punta Arenas, Chile, to the ice edge and back to Punta Arenas (Fig. 1). The whole research area is referred to as the Pacific region of the Southern Ocean. Water was collected at 4 stations (48, 55, 141 and 166, Fig. 1) and used in Experimental Series 48, 55, 141 and 166 (Table 1). Stns 48 and 55 are located north of the Polar Front (between 60.30° and 61° S), in the Polar Frontal Zone; Stns 141 and 166 are located south of the Polar Front in the Antarctic Ocean (Fig. 1). The hydrographic description of the research area will be provided by de Baar et al. (in press).

Sampling. During and after sample collection, all necessary precautions were taken to prevent trace metal contamination. After the arrival of 'Polarstern' at a station, trace metal sampling was always the first procedure to be executed. For the collection of water, a specially designed polyurethane-coated titanium frame equipped for 6 Go-Flo samplers of 12 l capacity each was used. The frame was lowered using a Kevlar wire to 200 m depth to be rinsed in the water column, after which it was lifted to the desired depth (30 to 60 m), depending on previously determined phytoplankton maximum density using flow cytometry. There, the bottles were tripped simultaneously with one large all-Teflon messenger Two casts were made per station. Upon arrival on deck the Go-Flos were mounted outside a clean, temperature-controlled laboratory container, in which the bottle enrichment experiments were performed. Rigorous use of clean techniques and clean equipment resulted in a close agreement of total iron concentration in the field and in the (control) incubation bottles. For example, during Series 166, total iron concentration in the control bottles was 0.33 ± 0.05 nM Fe (n = 3); in the field

(averaged over 0 to 100 m) 0.41 ± 0.11 nM (n = 4) was measured.

For enrichment experiments, polycarbonate incubation bottles (acid rinsed; 10 or 20 l) were used. The bottles were shipped in plastic bags, and opened only inside the clean container. Use of water from different Go-Flo bottles per incubation bottle ensured proper mixing of the water in each bottle. When resources permitted, 4 large (20 l) and 4 small (10 l) incubation bottles were filled. In this paper, the response of control and enriched (with 2 nM Fe as $FeCl_3$) large bottles receiving only white light are presented. Experiments using green and blue light are presented in van Leeuwe et al. (1998b).

Per series of experiments, 2 control and 2 enriched bottles were incubated at 80 µmol photons m^{-2} s^{-1} in 8 h light:16 h dark cycles and low temperature (2 to 5°C) conditions. Slow rotation (2 rpm) on rolling benches minimized sedimentation in the incubation bottles. During the 72 h experimental period, subsamples were collected from all individual bottles by pouring water into sampling vials in front of the clean air inflow of the clean container, thereby minimizing the possibility of contamination. At t = 0 h and t = 48 h a large sampling program was executed for analyses of nutrients, chl *a*, total Fe concentrations and phytoplankton cell characteristics (using flow cytometry: cell numbers, size classes, fluorescence). Every 12 h, samples for flow cytometry analyses were collected, and every 24 h samples for major nutrient and total Fe concentrations were collected. At t = 27 and t = 29 h samples were taken for stable isotope analyses (^{15}N), nitrate reductase activity and additional flow cytometry analysis. At 72 h, the remaining contents of the incubation bottles were used for protein and flavodoxin analyses.

Analytical procedures. Iron concentrations in individual experimental bottles were measured onboard using flow injection analysis followed by chemiluminescence detection (FIA-CL) (after Obata et al. 1993). In addition to routine sampling each 24 h, detailed studies of the fate of iron in control and enriched bottles were made during Series 141 and 166. Total (dissolvable) Fe was measured in unfiltered acidified (pH 1.6) samples and dissolved Fe was measured in 0.4 µm filtered samples. Immobilised 8-hydroxyquinoline on hydrophobic vinyl polymer was used for pre-concentration [manufactured according to the method of Landing et al. (1986)], and luminol was used as chemiluminescence reagent. The stability of the background signal and blank allowed reproducible analysis of even

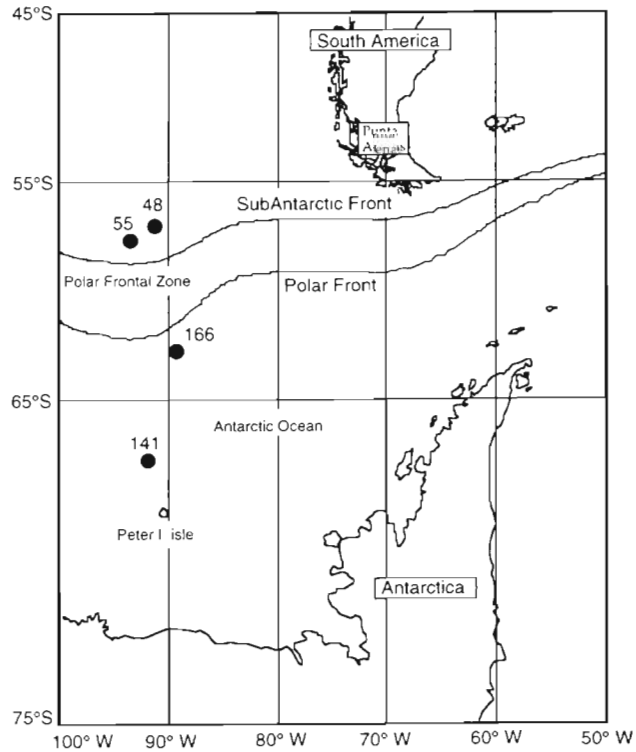

Fig. 1. Stations where water was sampled for iron enrichment experiments during the ANT XII/4 cruise aboard RV 'Polarstern' (March to May 1995), and approximate position of the major fronts after Whitworth (1988). The Southern Ocean is defined as all waters south of the Subantarctic Front. The Antarctic Ocean comprises all waters south of the Polar Front, and exhibits high concentrations of the major nutrients nitrate, phosphate and silicate (Table 2, Experimental Series 141 and 166). The in-between Polar Frontal Zone also has high nitrate and phosphate concentrations, but low silicate concentrations (Table 2, Experimental Series 48 and 55)

Table 1 Experimental series, bottle numbers, station numbers, date (April 1995), time, location and sampling depth for iron enrichment experiments

Series (bottles)	Stn no.	Date	Time	Location	Depth
Series 48 (13–16)	48	1 Apr	13:45 h	57° 15′ S, 91° 14′ W	60 m
Series 55 (21–24)	55	4 Apr	12:30 h	57° 53′ S, 93° 32′ W	40 m
Series 141(43–47)	141	20 Apr	21:00 h	67° 13′ S, 91° 50′ W	40 m
Series 166 (51–54)	166	26 Apr	13:45 h	62° 60′ S, 89° 30′ W	35 m

the lowest reported 0.05 nM concentration. For consecutive analyses of the same sample the reproducibility was within 5 % standard deviation (SD) over the whole 0.05 to 2 nM working range. A detection limit (3 × SD) of 0.015 nM during this expedition was determined. Accuracy was validated using reference seawater NASS-4 certified at 1.88 ± 0.29 nM. During this expedition we found 1.81 ± 0.15 nM (n = 4) (de Baar et al. in press).

Iron speciation during the incubation was measured in a separate experimental series with competitive ligand equilibration adsorptive cathodic stripping voltammetry using 1-nitroso-2-naphtol as competing ligand (Gledhill & van den Berg 1994). Full details on the analytical protocol, the data treatment and results of the field sampling programme are given in Nolting et al. (in press).

Stable isotope uptake experiments using ^{15}N-nitrate (all series) and ^{15}N-nitrate and ^{15}N-ammonium (Series 141 and 166) were performed at t = 27 and t = 29 h. The experimental bottles were supplemented with ^{15}N-nitrate and ^{15}N-ammonium enrichments of less than 10% of the ambient concentrations. Short-term ^{15}N incubations were performed over 3 to 5 h. Replicate 500 to 750 ml samples were filtered onto 11 mm diameter precombusted GF/F filters, after which the filters were retained and frozen for onshore analyses of ^{15}N content as well as particulate organic N and C. For these analyses, a Carlo Erba Elemental Analyser was used for conversion of the particulate organic nitrogen to N_2, followed by ^{15}N measurements with an Optima Stable Isotope Mass Spectrometer. The analyses also provided the particulate organic nitrogen (PON) concentrations in the experiments. Specific nitrate and ammonium uptake rates were calculated according to Dugdale & Goering (1967).

Shipboard nutrient analyses were performed on a continuous flow TRAACS 800 autoanalyser, according to methods of Grasshoff (1983), with the following precision: nitrate 0.20 µM, ammonium 0.10 µM, phosphate 0.05 µM, silicate 0.30 µM (<45 µM samples) and 0.80 µM (>45 µM samples). All nutrient measurements had coefficients of variation <1%. Samples were collected every 24 h, filtered through 0.45 µm pore size Acrodisc filters immediately, stored in a refrigerator at 4°C, and measured after the last samples had been collected at the end of each experimental series.

Nitrate reductase activities were determined onboard after Timmermans et al. (1994) in replicate 1000 ml samples between t = 29 and t = 32 h. The potential activity (i.e. under optimal conditions in cell extracts) is reported as the rate of nitrite formation.

Cell counts were made and size classes were determined at the beginning and end of the light period using a Coulter Counter flow cytometer. Algae smaller than 20 µm were discerned on the basis of differences in flow cytometric parameters (size and fluorescence) and combined into clusters. Cell counts were used to calculate net growth rates based on the model

$$N_t = N_0 e^{kt}$$

by linear regression on the log-transformed data (N = cell number at time 0 and time t and k is a constant).

Chl *a* concentrations were determined in preserved samples according to Holm-Hansen et al. (1965). Samples were collected at t = 0 and t = 48 h, filtered onto GF/F filters, and preserved at −20°C prior to analysis onshore. Chl *a* concentrations in the field were kindly provided by M. Templin and U. Bathmann (Alfred Wegener Institute, Bremerhaven, Germany).

Changes in cellular polypeptide composition were monitored by SDS PAGE (polyacrylamide gel electrophoresis). At the end of the experimental period (t = 72 h), cells were collected on GF/F filters and frozen until sample preparation in the laboratory. Filters were solubilized by grinding in sodium carbonate buffer containing lithium dodecyl sulphate and proteins were precipitated in cold TCA/acetone essentially as described by LaRoche et al. (1996). Precipitated proteins were solubilized in PAGE reducing buffer and separated on 15 % polyacrylamide gels. Variation in relative abundance of flavodoxin was determined from western blots of SDS PAGE gels probed with polyclonal antiserum raised against flavodoxin isolated from the diatom *Phaeodactylum tricornutum* (LaRoche et al. 1995) followed by incubation with goat anti-rabbit horseradish peroxidase. Immunoreactive polypeptides were detected by chemiluminescence and reaction products quantified by laser densitometry. In addition, blots were incubated sequentially with antiserum raised against RUBISCO isolated from the prymnesiophyte alga *Isochrysis galbana* (McKay et al. 1997, P. Falkowski unpubl.).

RESULTS

Throughout the cruise, HNLC conditions persisted, at least for the major nutrients nitrogen and phosphorus. In the surface water the initial nitrate concentrations varied between 19.9 and 27.5 µM (Table 2), whereas chl *a* concentrations never exceeded 0.2 µg l^{-1} (Fig. 2). Silicate concentrations in the surface water varied considerably north (<3.5 µM, Series 48 and 55) and south (>13 µM, Series 141 and 166) of the Polar Front (Table 2). Generally, there was good agreement between nutrient concentrations and chl *a* concentrations measured in the field and those measured in bottles at the beginning of each experimental series. Fe concentrations in surface waters varied, but rarely exceeded 0.5 nM (Fig. 3).

Major nutrients

At the start of the bioassays, nitrate concentrations were constant, ranging from 19.9 to 27.5 µM (Table 2). In contrast, silicate concentrations gave a clear indication of the differences in water masses sampled: Series 48 and 55, north of the Polar Front, had relatively low initial silicate concentrations (2.7 to 3.5 µM), whereas Series 141 and 166, south of the Polar Front, had much higher silicate concentrations (13 to 14 µM). During the bottle experiments, little or no change in major nutrient concentration was observed within the first 48 h (Table 2), both within each experimental treatment and between controls and Fe amended bottles.

Iron

Initial iron concentrations (Fig. 3) measured in control bottles matched well with concentrations in ambient surface waters (de Baar et al. in press). In control bottles, total iron remained constant and at low concentrations, supporting our assertion that no iron was introduced to bottles as a result of handling during the time series sampling

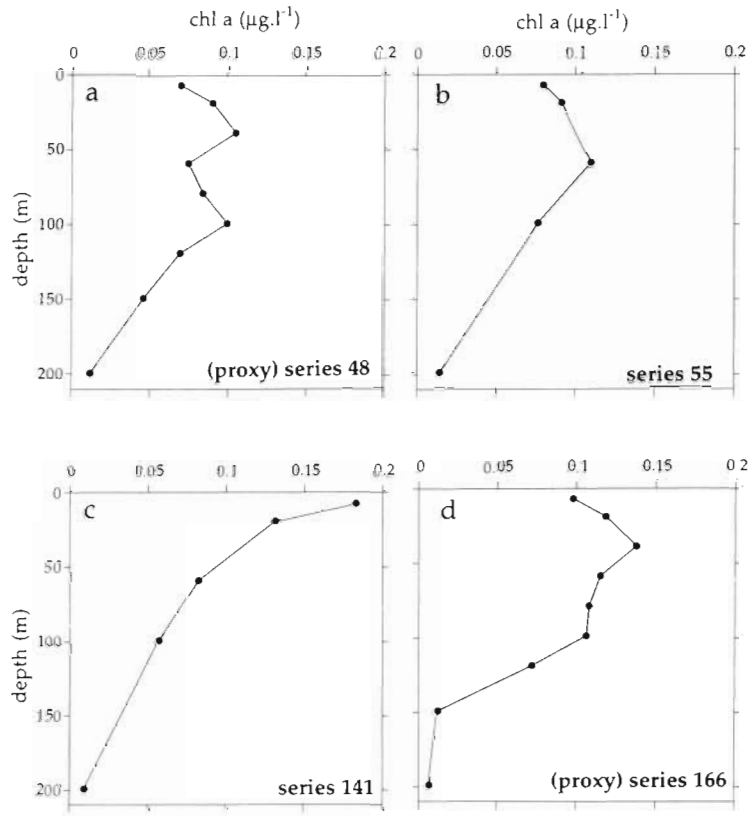

Fig. 2. Chl *a* concentration with depth at, or close to (proxy), stations where water for iron enrichment experiments was collected

Table 2. Average concentrations (µM) of ammonium, nitrate, silicate and phosphate during bottle experiments at t = 0, 24 and 48 h. Samples were taken at dawn; n = 4 except for Series 166 (n = 3). Nitrate (all series) and ammonium (Series 141 and 166) concentrations are corrected for addition of ^{15}N

	Control			Fe enriched		
	t = 0 h	t = 24 h	t = 48 h	t = 0 h	t = 24 h	t = 48 h
Series 48						
NH_4^+	1.23	1.18	1.16	1.23	1.25	1.25
NO_3^-	21.12	20.65	20.62	22.49	21.99	21.96
$H_4SiO_4^0$	3.49	3.50	3.49	3.49	3.49	3.50
PO_4^{3-}	1.51	1.51	1.51	1.53	1.53	1.51
Series 55						
NH_4^+	0.70	0.67	0.67	0.70	0.67	0.70
NO_3^-	19.85	19.57	19.58	21.38	21.16	21.21
$H_4SiO_4^0$	2.78	2.83	2.80	2.74	2.76	2.78
PO_4^{3-}	1.42	1.42	1.41	1.42	1.42	1.42
Series 141						
NH_4^+	0.85	0.86	0.81	0.89	0.78	0.78
NO_3^-	24.72	24.70	24.71	26.08	26.20	26.10
$H_4SiO_4^0$	14.22	14.29	14.24	14.19	14.23	14.23
PO_4^{3-}	1.73	1.74	1.73	1.72	1.73	1.73
Series 166						
NH_4^+	0.94	0.90	0.88	0.96	0.99	0.92
NO_3^-	26.01	26.02	25.90	27.51	27.37	27.42
$H_4SiO_4^0$	13.76	13.90	13.67	13.75	13.96	13.90
PO_4^{3-}	1.85	1.85	1.85	1.86	1.85	1.85

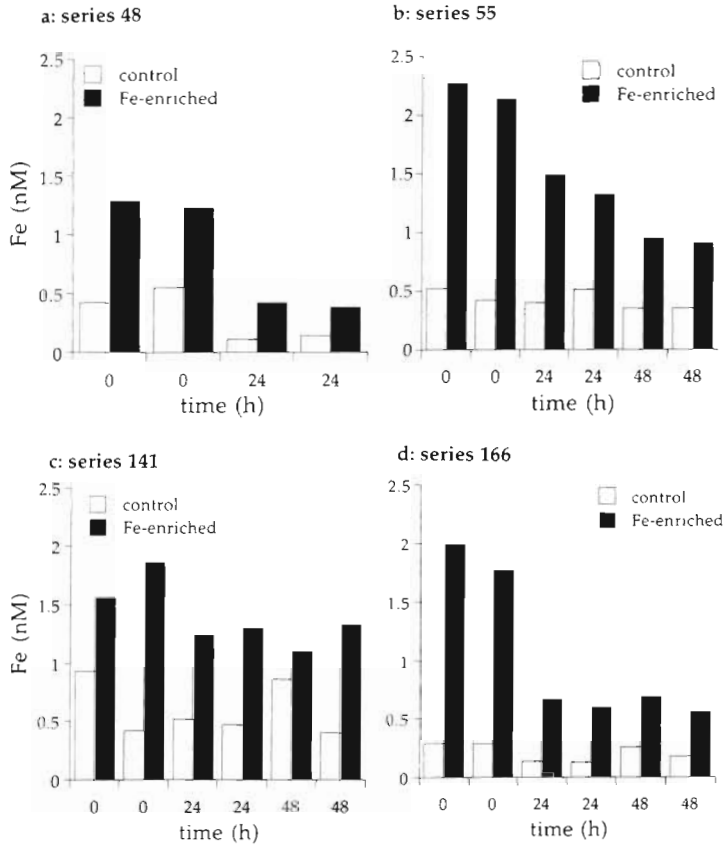

Fig. 3. Total Fe concentrations over time in duplicate control and Fe enriched incubation bottles during the experimental period. During Series 141, samples were taken from separate (parallel, identical conditions) 500 ml polycarbonate bottles

operations. The 2 higher values in the duplicates at t = 0 and t = 48 h in Series 141 are regarded as exceptions. Iron concentrations in amended bottles were close to the intended 2 nM addition, with values of total iron immediately after addition ranging from 1.2 to 2.2 nM (Fig. 3). In the course of most of the bioassays, a steady decrease in total iron concentrations was observed, whereas in Series 141 little variation was seen.

During Series 141 and 166, the fate of both total and dissolved iron was followed in detail (Fig. 4). In the course of each of these experimental series, total and dissolved iron concentrations in the control bottles showed variation. Remarkable is the peak in dissolved Fe at the beginning of the dark period (t = 56 h). In iron enriched bottles, a decrease in the total (Series 141 and 166) and dissolved (Series 141) iron was observed. As in the control bottle, a peak in dissolved iron was measured at t = 56 h. After 72 h, total and dissolved iron concentrations in enriched and control bottles were still different, with the highest concentrations measured in enriched bottles.

Analyses with adsorptive cathodic stripping voltammetry in a separate experimental series (Series 198; total dissolved Fe 0.36 nM; Table 3, no other data shown) gave conditional stability constants (log K') of 20.8 to 21.15 and demonstrated that relatively high levels of organic ligands (L_t) (range: 5.0 to 9.3 nEq. of M Fe) were present (Table 3). Resulting calculations of the organic α ($K' \times [L_t]$), as a measure of organic complexation, were similar to those found by Rue & Bruland (1995), Gledhill & van den Berg (1994) and van den Berg (1995). Little variation was found in the parameters measured during the experimental period. In combination with the measured total and dissolved iron concentrations (in the subnanomolar range), it was deduced that 80 to 90% of the iron was organically complexed. Similar findings of high capacity organic ligand complexation were noted in the sampling regime dedicated to natural organic complexation of iron in the research area (Nolting et al. in press).

Chlorophyll *a*

The lowest chl *a* concentrations were measured in Series 166 (Fig. 5). These values were lower than those measured in the field close to the station where the water for Series 166 was collected (Fig. 2d). Over the course of the experiments, small increases (Series 48, 55, 141) or slightly lower concentrations of chl *a* were measured. No significant differences (t-test, $p > 0.1$) were observed between control and enriched bottles over the first 48 h.

Cell numbers, size classes and growth rates

Generally, low numbers of phytoplankton were present. In Series 48 and 55 the highest initial numbers were observed: cell numbers were much lower in Series 141 and 166. During the experimental period, no clusters other than those already present at the beginning of the experiment were observed. This is seen as an important indication that no significant shift in species composition took place.

In Series 48, flagellates and small dinoflagellates were the main phytoplankters (van Leeuwe et al. 1998b). Three clusters were distinguished (Fig. 6); Clusters I and II were present with relatively high and comparable numbers, whereas Cluster III was present

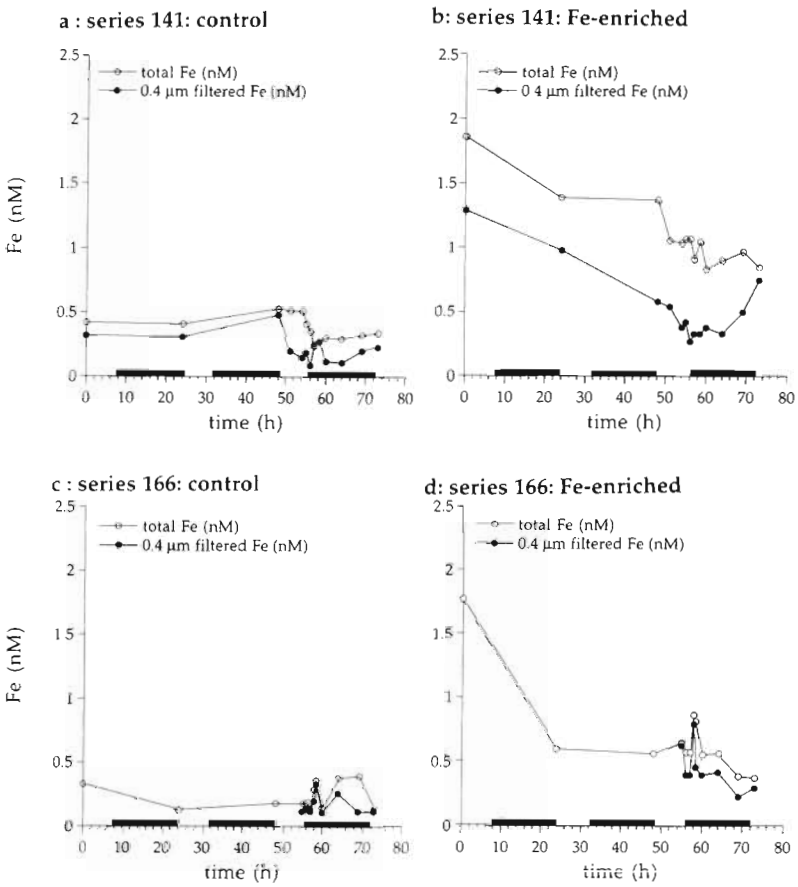

Fig. 4. Total and dissolved Fe concentrations in control and Fe enriched incubation bottles from Series 141 and 166. Dark periods are indicated by black bars on the *x*-axis. In Series 166, dissolved Fe measurements started at $t = 48$ h

at low numbers. Initial total cell numbers were low: 7000 to 8000 ml^{-1}. This increased nearly 2-fold, with no significant differences between control and Fe enriched bottles. Growth rates (total cell numbers: I + II + III) of 0.22 d^{-1} for the controls and 0.23 d^{-1} for Fe enriched bottles were calculated.

Table 3. Time series of conditional stability constants (K') and concentrations of dissolved organic ligand (L_t, in nEq. of M Fe) calculated from competitive ligand equilibration/adsorptive cathodic stripping voltammetry titration data in a control bottle in an additional experimental series (Series 198; no other data shown). Calculations according to Nolting et al. (in press)

	$t = 0$ h	$t = 24$ h	$t = 48$ h
L_t (nEq.)	7.5 ± 0.3	5.0 ± 0.3	9.3 ± 0.5
Log K'	21.15 ± 0.15	20.87 ± 0.17	20.80 ± 0.11
Log α (organic) ($K' \times [L_t]$)	13.03	12.57	12.77

In Series 55 only 1 cluster of algae was discerned by flow cytometry (Fig. 7). As in the previous series, initial cell numbers were low (7000 to 8000 cells ml^{-1}), with little variation between experimental treatments. This was reflected in the calculation of growth rates: 0.21 d^{-1} for control bottles and 0.22 d^{-1} for enriched bottles over the period 24 to 72 h. The high frequency sampling programme indicated a smooth increase in cell numbers throughout the experimental period following an initial lag phase of 48 h. Microscopic identification demonstrated that flagellates and small dinoflagellates were the dominant phytoplankters (van Leeuwe et al. 1998b).

For Series 141 and 166, low initial total cell numbers were measured (1500 cells ml^{-1}). For Series 141, little or no change in cell numbers was observed throughout the experimental period (Fig. 8). Of the 3 clusters discerned, only Cluster IV cell numbers increased slightly. No differences between experimental treatments were observed within the 72 h experimental period. This is reflected in the growth rates calculated over the 24 to 72 h period: 0.11 d^{-1} for controls and 0.12 d^{-1} for Fe enriched bottles. Microscopic identification showed that this experimental series was the only series of the 4 discussed here to contain significant numbers of diatoms. *Fragilariopsis kerguelensis*, *Coscinodiscus* sp. and *Thalassiosira* sp. were, next to small flagellates, the dominant phytoplankton, comprising 50 to 60% of the total chl *a* (van Leeuwe et al. 1998b).

For Series 166, 3 different clusters were discerned by flow cytometry (Fig. 9). Of these, only cells of Cluster VII showed a steady increase over the experimental period. No differences in treatments were observed. Growth rates calculated from total cell numbers over the 24 to 72 h period were 0.14 d^{-1} for controls and 0.10 d^{-1} for enriched bottles. The high frequency sampling program again, as in Series 55, showed an increase in cell numbers after approximately 60 h. Flagellates and small dinoflagellates dominated the phytoplankton assemblage (van Leeuwe et al. 1998b).

Nitrogen uptake and metabolism

Specific nitrate and ammonium uptake rates were low. For each experimental series, rates of ammonium

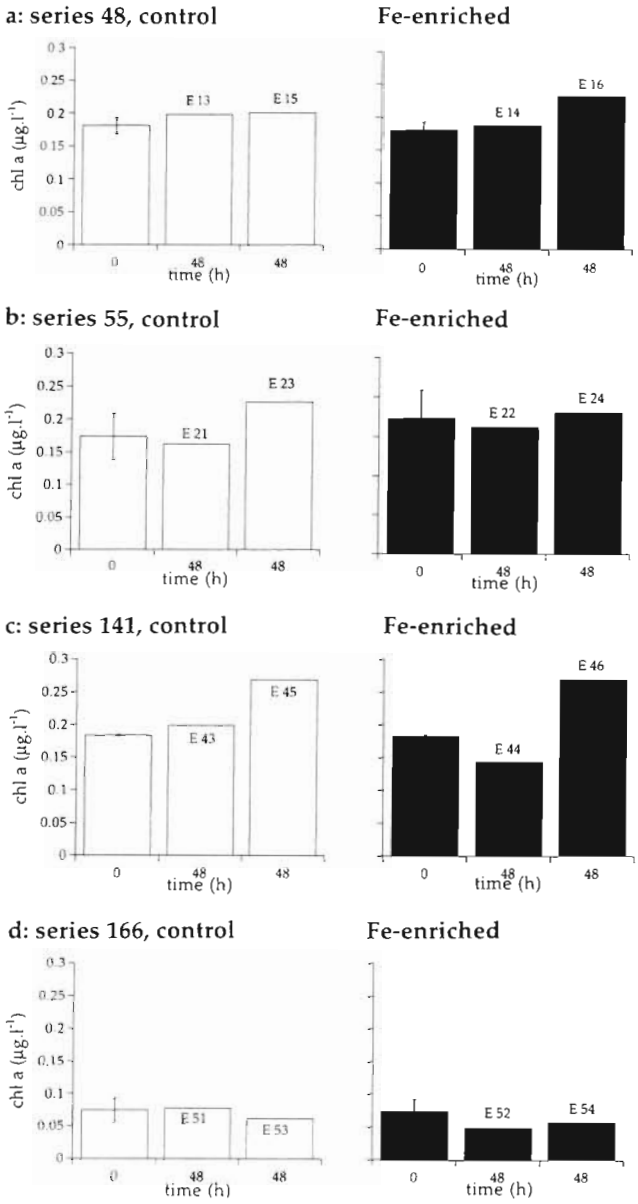

Fig. 5. Chl *a* concentrations in control and Fe enriched incubation bottles at t = 0 h (average, mixed sample) and t = 48 h (individual bottles). Individual bottle numbers are indicated. Error bars show SD

uptake were substantially higher than rates of nitrate uptake; obviously, regenerated production prevailed over new production. Higher specific nitrate uptake rates were observed in Fe enriched bottles as compared to controls (Table 4). In contrast, ammonium uptake rates were comparable in control and Fe enriched samples. Specific rates of nitrate uptake measured in Series 141 and 166 were somewhat lower than those found in Series 48 and 55.

The *f*-ratios [nitrate uptake/(nitrate + ammonium uptake)] in the controls varied from 0.10 in Series 166 to 0.22 in Series 141, the latter in keeping with the presence of diatoms in this experiment. The *f*-ratios in the Fe enriched samples (0.15 and 0.23 in Series 166 and 141, respectively) were higher than in the controls.

The above described differences in nitrate uptake rates were reflected in nitrate reductase activity assays (Table 5). The potential nitrate reductase activity in enriched samples was higher than in control samples. The potential activity was higher than the uptake rates measured with ^{15}N, but the general trend was similar.

Immunoblotting

Due to the low biomass encountered in the HNLC waters sampled, results of Western immunoblotting are not available for Series 48 and 166. For Series 55 and 141, however, flavodoxin staining was observed in samples taken from both control and enriched bottles (Fig. 10). For Series 55, flavodoxin staining was weak and was not observed in all trials. In each case, intensity of flavodoxin staining was higher in enriched samples relative to controls, even when staining was normalized to RUBISCO (Fig. 10b).

DISCUSSION

The HNLC conditions encountered in the Pacific region of the Southern Ocean allowed us to further address the role of iron in phytoplankton physiology. Assuming a cellular N:Fe molar ratio of 10 000 (Morel et al. 1991b), it is clear that the low levels of iron encountered (<0.5 nM) are not sufficient for a full consumption of available surface water nitrate. Similarly, nitrate levels encountered in these waters (>20 µM) should yield approximately 20 µg chl *a* l^{-1}, yet measured concentrations of chlorophyll in the experimental area fell consistently below 0.2 µg l^{-1}. The description 'High-Nutrient, Low-Chlorophyll' did not fully apply with respect to the nutrient silicate. In the northern part of the research area (Polar Frontal Zone, Series 48 and 55), silicate concentrations were relatively low, thereby likely posing an additional limitation on one (important) group of the phytoplankton community, the diatoms.

The long-term effects of iron addition on phytoplankton under HNLC conditions have been well documented (de Baar et al. 1990, Buma et al. 1991, Martin et al. 1994, Boyd et al. 1996, van Leeuwe et al. 1997, de Baar & Boyd in press). Short-term effects have received less attention. In the present short-term study, we found evidence indicating that at least some of the indigenous phytoplankton of the Pacific region of the Southern Ocean exist with an iron deficit. Phytoplankton sampled

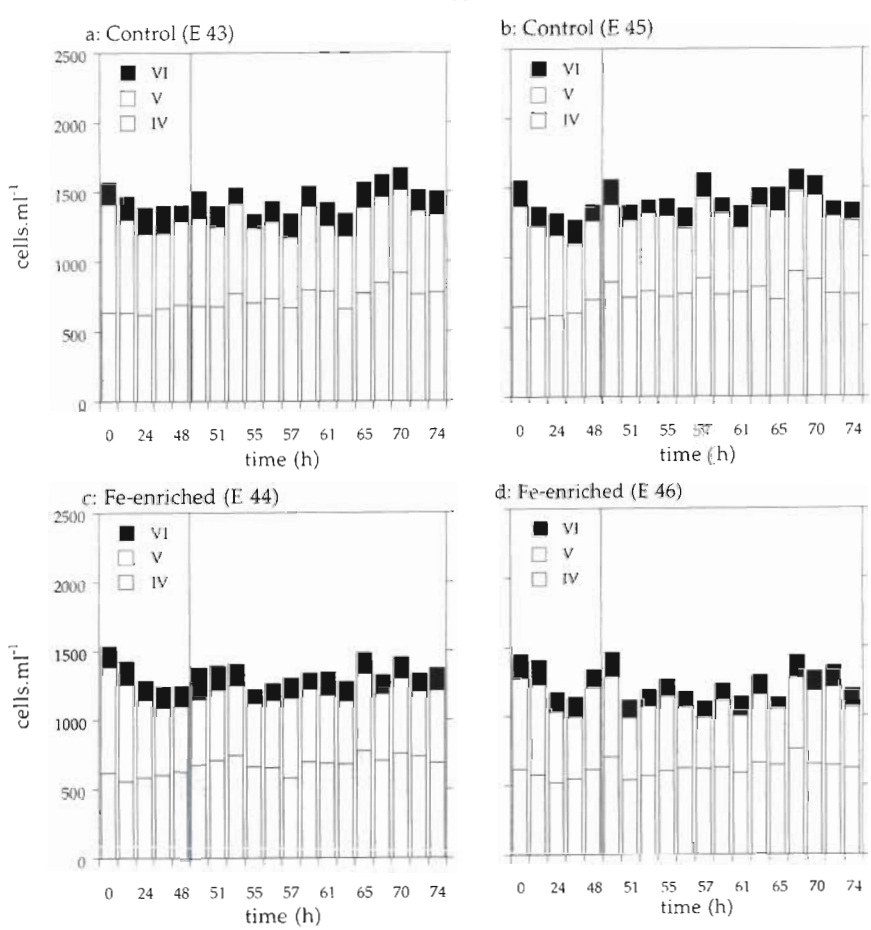

Fig. 8. Phytoplankton (<20 µm) cell numbers and flow cytometer Clusters IV, V and VI during Series 141 in control (bottles E 43 and E 45) and Fe enriched (bottles E 44 and E 46) incubations. Note the change in time scale after $t = 48$ h

biomass of the phytoplankton community'. This assumption (phytoplankton controls zooplankton) provides a different interpretation of the usual role (zooplankton controls phytoplankton) of grazers in HNLC conditions. For example, Geider & LaRoche (1994) point towards 3 factors which might be indicative of the importance of grazing control under HNLC conditions. First, chl *a* concentrations could be held constant through intense grazing. Alternatively, it is possible that chl *a* concentrations remain low due to constraints imposed by low iron levels (this study). Second, chl *a* increases after iron addition are mainly caused by an increase of the larger phytoplankton. Alternatively, it is hypothesized that a functional response of the microzooplankton (i.e. consuming the increase in the predominant indigenous phytoplankton following relief from their limitation) could mask the increase in the small phytoplankton: the large phytoplankton could increase through inadequate grazer response. Third, growth rates for small phytoplankton are reported to be 0.5 to 1.0 d^{-1}. Typical maximum growth rates for Antarctic phytoplankton are reported in a similar range, from 0.32 to 1.33 d^{-1} (Spies 1987, Sommer 1989). The results in the present study show that net growth rates are in the low end of this range, between 0.10 (Series 166) and 0.23 d^{-1} (Series 48).

Previously, it has been demonstrated that micro-zooplankton grazing activity can be vigorous in the marginal ice zone of the Bellingshausen Sea. Burkill et al. (1995) calculated a 3 to 40% daily turnover of phytoplankton in the spring. These results are not confirmed in the present study. Grazing experiments using the Landry & Hasset (1982) dilution method did not support intense grazing activity (data not shown). With the low phytoplankton numbers found in the research area, it is questionable whether any response can be observed. With initial cell numbers only in the few 1000 ml^{-1} range it is extremely difficult to obtain reproducible results in dilution series (B. R. Kuipers, NIOZ, The Netherlands, pers. comm.). At present, we have no explanation for the paradox of low grazing activity as measured during the experiments and the relatively high ammonium concentrations. Either we have underestimated zooplankton grazing, or ammonium comes from other sources such as phytoplankton lysis or bacterial ammonium production.

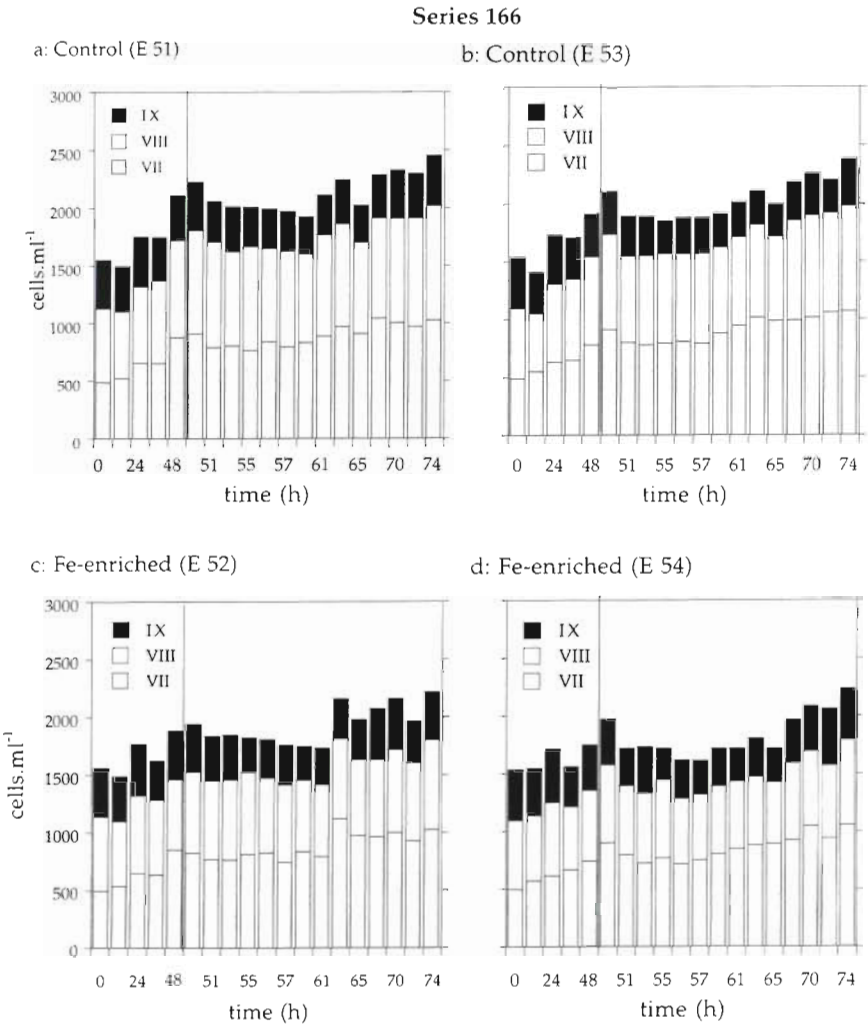

Fig. 9. Phytoplankton (<20 µm) cell numbers and flow cytometer Clusters VII, VIII and IX during Series 166 in control (bottles E 51 and E 53) and Fe enriched (bottles E 52 and E 54) incubations. Note the change in time scale after $t = 48$ h

The close agreement between iron concentrations in the field and in control incubation bottles is regarded as proof of contaminant-free sampling and incubation procedures. The *in situ* iron concentrations showed a weak trend towards elevated concentrations at stations closer to the Antarctic continent. For the oceanic stations, low dissolved iron concentrations were measured (0.05 to 0.5 nM, de Baar et al. in press); these values are somewhat lower than the iron concentrations previously reported in Drake Passage (0.1 to 0.7 nM, Martin et al. 1990). In the time series of total and dissolved Fe, it was demonstrated that concentrations varied (Fig. 4). Apart from the decreasing trends in concentrations through time, it was clear that unexpected peaks were also present. Particularly interesting were the elevated concentrations in dissolved Fe measured at the beginning of the dark period. This peak coincides with an apparent elevated activity of cell division. For Fe enriched bottles, the eventual decrease in iron resulted in concentrations after 24 to 48 h only slightly higher than those measured in control bottles. The rapid decline in iron concentrations has to be ascribed to (1) uptake by the micro-organisms, (2) adsorption to particulate matter (including the bottle wall) and (3) transfer of iron into pools not assessed in the total iron measurement (acidification to pH 1.6).

The results of competitive ligand equilibrations corroborate the findings by others (Gledhill & van den Berg 1994, Rue & Bruland 1995, van den Berg 1995, Wu & Luther 1995). The high concentrations of natural organic ligands measured should further decrease the availability of iron for the phytoplankton, assuming that only the non-complexed iron is available for the phytoplankton. The amount of free Fe (III) will be far below that required for unlimited phytoplankton growth in the Pacific region of the Southern Ocean. Takeda & Obata (1995) concluded that at least 0.8 nM Fe should be available in order to meet Fe requirements of large- (>10 µm) and medium-sized (3 to 10 µm) phytoplankton. Based on theoretical considera-

tions, Morel et al. (1991b) concluded that, below a concentration of 0.05 nM iron, only large phytoplankton are Fe limited. With the high organic complexation of iron as observed in the present study, maximum inorganic Fe concentrations of 0.06 nM can be calculated, assuming an inorganic α of $10^{11.9}$.

Given the small amount of iron added, coupled with its observed decline in bottles, it is not plausible that phytoplankton iron stress was fully relieved during the experimental period. The lowest ligand concentration measured was 5.0 nEq of M Fe. With background Fe concentrations of 0.1 to 0.4 nM, 2 nM Fe additions should theoretically be complexed completely by the available ligands. In the light of this, it is not surprising that flavodoxin persists in iron-amended samples. LaRoche et al. (1995) observed that flavodoxin levels were depressed only after enough iron had been added to account for a reduction of nitrate by phytoplankton. Similarly, McKay et al. (1997) found flavodoxin still expressed in 2 species of diatoms at 10 nM Fe. Clearly, Fe stress was not relieved in the present study. That flavodoxin levels not only persisted in enriched bottles but actually increased is somewhat curious, but may be explained by small shifts in community composition in favour of diatoms, especially large-sized cells not detected by flow cytometry, 72 h following iron amendment. Diatoms are particularly responsive to addition of iron to HNLC waters (Boyd et al. 1996, Coale et al. 1996, Zettler et al. 1996, van Leeuwe et al. 1997, de Baar & Boyd in press). That staining for flavodoxin was most intense in Series 141 (Antarctic Ocean) is consistent with the observation that diatoms (*Fragilariopsis kerguelensis*, *Coscinodiscus* sp., *Thalassiosira* sp.) were present in the phytoplankton assemblage during this experiment. A situation in which diatoms are iron-stressed, yet underrepresented in control bottles relative to enriched bottles, might explain the observed pattern of staining. Regardless, persistence of a flavodoxin signal in iron-amended bottles supports an iron-stressed status for diatoms in the study area.

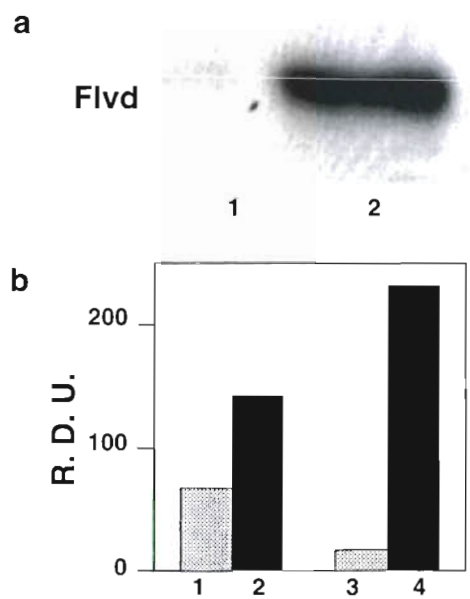

Fig. 10. (a) Western immunoblot analysis of phytoplankton from Series 141. The control sample (lane 1) and sample amended with 2 nM Fe (lane 2) were reacted with polyclonal antisera directed against diatom flavodoxin. Reactive bands were detected by chemiluminescence and quantified by laser densitometry. (b) Histogram derived from densitometric analysis of staining by anti-flavodoxin for Series 55 (lanes 1, 2) and 141 (lanes 3, 4). Control (shaded bars; lanes 1, 3) and iron-amended samples (black bars; lanes 2, 4) are shown. Antibody staining is normalized to RUBISCO staining from the same immunoblot and is presented in relative densitometer units (R.D.U.)

In summary, we have 2 lines of evidence which indicate that the indigenous phytoplankton of the Pacific region of the Southern Ocean, as investigated in the austral autumn, is iron-stressed. First, upon addition of iron, short-term responses in nitrogen assimilation were observed. Within 28 to 32 h, significant enhancement in nitrate uptake and elevated levels of nitrate reductase activity were measured. Given the absence of short-term shifts in species composition, the changes in N metabolism are indicative of iron stress in the indigenous phytoplankton assemblage (flagellates). A response in diatoms is only observed when silicate concentrations are well above the half-saturation constants for growth (>10 µM, Antarctic Ocean). Second, flavodoxin was present in samples of the indigenous diatom community, which is indicative of iron stress in diatoms endemic to the HNLC waters.

Based on these observations, we conclude that through bottom-up (i.e. ecophysiological) control, iron plays an important role in the Pacific region of the Southern Ocean. Iron stress may result in low numbers of small-sized phytoplankton and thereby limit microzooplankton numbers. A combination of silicate and iron stress prevents diatom dominance north of the Polar Front.

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